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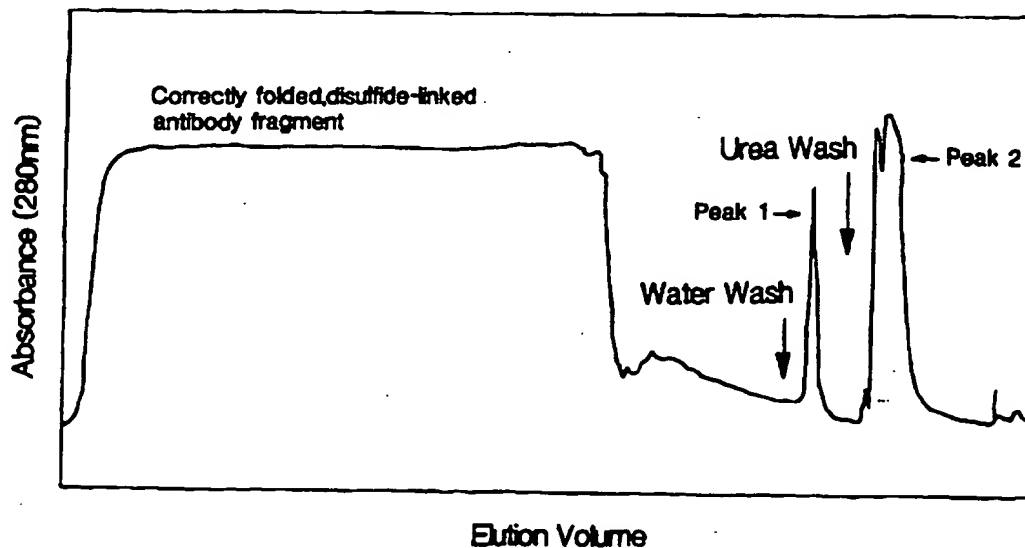
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(54) Title: ANTIBODY PURIFICATION BY LOW-PH HYDROPHOBIC INTERACTION CHROMATOGRAPHY



(57) Abstract

A process for purifying an antibody is provided. In this process, a mixture containing the antibody and a contaminant is subjected to low pH hydrophobic interaction chromatography (LPHIC) at low salt concentration. The antibody is eluted from the column in the fraction which does not bind thereto. This process can be preceded and followed by other purification steps.

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ANTIBODY PURIFICATION BY LOW-PH HYDROPHOBIC INTERACTION CHROMATOGRAPHY

BACKGROUND OF THE INVENTIONField of the Invention

This invention relates generally to antibody purification. In particular, the invention relates to a
5 method for recovering an antibody fragment from variants, impurities, and contaminants associated therewith.

Description of Related Art

Hydrophobic interaction chromatography (HIC) is a useful tool for separating molecules based on
their hydrophobicity. Generally, sample molecules in a high salt buffer are loaded on the HIC column. The
10 salt in the buffer interacts with water molecules to reduce the solvation of the molecules in solution, thereby
exposing hydrophobic regions in the sample molecules which are consequently adsorbed by the HIC column.

The more hydrophobic the molecule, the less salt needed to promote binding. Usually, a decreasing salt
gradient is used to elute samples from the column. As the ionic strength decreases, the exposure of the
hydrophilic regions of the molecules increases and molecules elute from the column in order of increasing
15 hydrophobicity. Sample elution may also be achieved by the addition of mild organic modifiers or detergents
to the elution buffer. HIC is reviewed in Protein Purification, 2d Ed., Springer-Verlag, New York, pgs 176-
179 (1988).

HIC has been used by various researchers for purification of antibodies. Danielsson *et al.*, Journal
of Immunological Methods 115:79-88 (1988) found that HIC was particularly useful for purification of
20 monoclonal antibodies from mouse ascites when the isoelectric point of the antibodies was below 7.2. HIC
was performed with an Alkyl Superose HRTM column. The buffer system was 0.1M phosphate, with addition
of ammonium sulfate. Usually the starting buffer contained 2M ammonium sulfate. Bridonneau *et al.*, Journal
of Chromatography 616:197-204 (1993) were interested in determining whether or not different HIC columns
could be used for selective purification of human immunoglobulin G (IgG) subclasses. The antibodies were
25 adsorbed on Phenyl-, Butyl-, or Octyl-SepharoseTM columns in 1M ammonium sulfate (pH 7.0) and eluted
with decreasing salt gradient. Octyl-SepharoseTM medium yielded a poorly adsorbed fraction somewhat
enriched in IgG_{2a}. See also Berkowitz *et al.*, Journal of Chromatography 389:317-321 (1987); Gagnon *et al.*
(90th Annual Meeting, American Society for Microbiology, Anaheim, May 13-17, 1990) Abstract No. 0-4;
Johansson *et al.* Biol. Recombinant Microorg. Anim. Cells, (Oholo 34 Meeting), 409-414 (1991); Pavlu *et al.*,
30 Journal of Chromatography 359:449-460 (1986) and Abe *et al.*, Journal of Biochemical and Biophysical
Methods 27:215-227 (1993) concerning HIC of antibodies.

HIC has also been used for purifying antibody fragments. Inouye *et al.*, Protein Engineering, pgs 6,
8 and 1018-1019 (1993); Inouye *et al.*, Animal Cell Technology: Basic & Applied Aspects 5:609-616 (1993);
Inouye *et al.*, Journal of Biochemical and Biophysical Methods 26:27-39 (1993) and Morimoto *et al.*, Journal
of Biochemical and Biophysical Methods 24:107-117 (1992) prepared F(ab')₂ fragments from pepsin digests
35 of mouse IgM monoclonal antibodies using a TSKgel Ether-5PWTM HIC column. The antibody fragments
were salted out with 60% ammonium sulfate and the precipitates were dissolved into phosphate-buffered saline
(PBS, pH7.4) containing 1M ammonium sulfate. This solution was loaded onto the HIC column which had
been equilibrated with PBS also containing 1M ammonium sulfate. The F(ab')₂ fragments which were

adsorbed onto the column were eluted by reducing the ammonium sulfate concentration in the elution buffer to 0M. Inouye *et al.* found that the fraction containing the $F(ab')_2$ was homogeneous by both SDS-PAGE and gel filtration HPLC. The method was considered to be suitable for large-scale purification of $F(ab')_2$ fragments. Similarly, Rea *et al.*, *Journal of Cell. Biochem. Suppl.* 0, Abstract No. X1-206 (17 Part A), p.50
5 (1993) evaluated HIC for purification of a $F(ab')_2$ fragment produced by peptic digestion of a murine IgG_{2a} monoclonal antibody. Protein A purification for removal of residual intact antibody preceded the HIC step. The purification performance of three different HIC columns was tested at several different salts and pHs. POROS PETM (Phenyl ether) was found to be the best column and phosphate-buffered sodium sulfate at pH 8 gave the best resolution of the $F(ab')_2$ fragment.

10

SUMMARY OF THE INVENTION

In contrast to the above described HIC techniques, which are generally performed at about neutral pH in the presence of high salt concentrations (using a salt gradient to elute the antibody), the instant invention relates to low pH hydrophobic interaction chromatography (LPHIC) for antibody purification. Preferably, the
15 LPHIC is performed at low salt concentrations, *i.e.*, about 0-0.25M salt, preferably about 0-0.1M salt and more preferably 0-50mM salt. This low salt concentration also applies to the loading buffer. Preferably, no salt gradient is used to elute the antibody.

In particular, the invention provides a process for purifying an antibody from a contaminant which comprises loading a mixture containing the antibody and the contaminant on a hydrophobic interaction
20 chromatography column and eluting the antibody from the column with a buffer having a pH of about 2.5-4.5. Preferably the buffer is at a pH of about 2.8-3.5 and more preferably at a pH of about 3.1. Usually, the mixture loaded onto the column is at about the same pH as the elution buffer.

The method is particularly useful for purifying antibody fragments, especially correctly folded and disulfide linked antibody fragments (*e.g.* Fab fragments) from contaminating antibody fragments which are not
25 correctly folded and/or disulfide linked. The invention resides, at least in part, in the identification of a problem associated with the formation of recombinant immunoglobulins. It has been observed that such production results in the formation of functional $F(ab')_2$ antibodies as well as a variety of incorrectly associated light and heavy fragments. The most difficult impurity to remove has been characterized herein as a correctly folded antibody fragment whose light and heavy chains fail to associate through disulfide bonding. This
30 antibody impurity can be detected by SDS PAGE gels and Reverse Phase HPLC as heavy and light chains. The LPHIC described herein provides a means for substantially removing this contaminant from partially purified compositions derived from host cells producing the recombinant antibody fragment, although it is not limited to purification of recombinant products.

The invention also relates to the antibody formulation prepared by the process and uses for this
35 antibody formulation.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a typical flow through chromatogram of low pH hydrophobic interaction chromatography (see Example 1). The column was operated in the flow-through mode. Correctly folded, disulfide linked antibody fragment flows through. Light chain, heavy chain, light-heavy aggregates and non disulfide linked antibody fragment species remain attached to the column. Peak 1 is the flow-through following water wash, peak 2 is the urea wash of the bound species.

Fig. 2 depicts a Reverse Phase HPLC analysis of ABXTM and Phenyl SepharoseTM Fast Flow (FF) pools of anti-CD18 MHM23 antibody fragment. Chromatogram 1; ABXTM pool containing light and heavy chain contaminants present before LPHIC purification. Chromatogram 2; LPHIC purification. Chromatogram 3; Reverse Phase analysis of the column regeneration buffer containing light and heavy chain impurities and antibody fragments retained by the Phenyl SepharoseTM Fast Flow column.

Figs. 3A-3D depict near UV and far UV spectra of two antibody fragments, rhuMAb H52OZG1 and rhuMAb H52OZG2, obtained by circular dichroism. Fig. 3A depicts near-UV spectra of rhuMAb H52OZG2 and Fig. 3B depicts far-UV spectra of rhuMAb H52OZG2. This antibody is a mutant of rhuMAb H52OZG1 in which cysteine residues 215 and 228, involved in disulfide bonding between the heavy and light chains, were mutated to serine residues. Circular dichroism spectra in both the far and near UV regions showed a transition point around pH 3.2 (thick line). A transition point represents a change from folded antibody fragment, to its unfolded state. Fig. 3C is a near-UV spectra of rhuMAb H52OZG1 and Fig. 3D is a far-UV spectra of rhuMAb H52OZG1. This antibody fragment showed a different transition point at pH 2.5 (thick line).

Fig. 4 is a bar graph depicting the consequences on product yield of varying the HIC pH. ABXTM purified antibody fragment pools containing the linkless antibody impurity (*i.e.* having no disulfide bond between the heavy and light chain) were further purified on a Phenyl SepharoseTM Fast Flow column. The purification was performed at pH values between 3.0 and 6.5 in order to determine the best pH to obtain maximum yield and purity. Analysis of the flow through pools was performed using Reverse Phase HPLC. From the bar graph it can be seen that pH 3.1 represents the best value to maximize both purity and yield in the purification of rhuMAb H52OZG1 antibody fragment.

Figs. 5A and 5B depict L-F(ab')₂ design and expression cassette described in Example 2 herein. Fig. 5A is a schematic representation of L-F(ab')₂ variants (v1, v2 and v3) in which variable (V) domains from the anti-p185^{HER2} Ab, huMAb4D5-8, and from the anti-CD18 Ab, huMAb H52OZG1, are denoted by open and filled boxes, respectively. Fig. 5B is a schematic representation of the dicistronic operon for expression of anti-p185^{HER2} L-F(ab')₂ variants derived from plasmid pAK19. Expression is under the transcriptional control of the *E. coli* alkaline phosphatase promoter (*phoA*) which is inducible by phosphate starvation. Each antibody (Ab) chain is preceded by the *E. coli* heat-stable enterotoxin II (stII) signal sequence to direct secretion to the periplasmic space of *E. coli*. The humanized V_L and V_H (both copies) domains are precisely fused on their 3' side to human κ_1 C_L and IgG₁ C_{H1} constant domains, respectively. The H chain comprises tandemly duplicated segments in which the 5' C_{H1} domain is joined in frame to a V_H encoding segment. The 3' C_{H1} domain is followed by the bacteriophage lambda λ_{10} transcriptional terminator (*ter*).

Figs. 6A-6C depict FPLC size exclusion chromatography analysis of anti-p185^{HER2}. Fig. 6A shows L-F(ab')₂ v1; Fig. 6B shows thioether linked F(ab')₂ and Fig. 6C shows Fab titration with p185^{HER2} extracellular domain (ECD).

Fig. 7 shows inhibition of proliferation of BT474 cells by anti-p185^{HER2} L-F(ab')₂, F(ab')₂ and Fab fragments. Data shown are presented as a percentage of results with untreated cultures (mean of duplicate measurements and representative of three separate experiments). Monovalent and bivalent fragments as judged by titration with p185^{HER2} ECD and gel filtration, are represented by open and closed symbols, respectively.

Fig. 8 depicts pharmacokinetics of anti-p185^{HER2} L-F(ab')₂ v1, Fab and thioether-linked F(ab')₂ fragments in normal mice. Serum samples were recovered from groups of 45 female CD-1 mice after a single tail vein injection (10 mg/kg). The mean serum concentrations ($C_t \pm SD$) estimated by antigen (Ag)-binding ELISA are shown together with the non-linear least squares fits (—):

$$C_t = 155e^{-1.73t} + 190e^{-0.042t}, \text{ L-F(ab')}_2 \text{ v1};$$

$$C_t = 239e^{-0.704t} + 38.4e^{-0.264t}, \text{ F(ab')}_2;$$

$$C_t = 440e^{-4.99t} + 2.69e^{-0.442t}, \text{ Fab.}$$

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions:

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies (including agonist and antagonist antibodies), polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, generally the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies (see Example 2 below); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567 [Cabilly *et al.*]). The "monoclonal

antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, Nature, 352:624-628 (1991) and Marks *et al.*, J. Mol. Biol., 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly *et al.*, *supra*; Morrison *et al.*, Proc. Natl. Acad. Sci. USA, 81:6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see: Jones *et al.*, Nature, 321:522-525 (1986); Reichmann *et al.*, Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992). The humanized antibody includes a PrimatizedTM antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) on the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Holliger *et al.*, Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993).

As used herein, "buffer" refers to a buffered solution that resists changes in pH by the action of its acid-base conjugate components. The buffer for the hydrophobic interaction chromatography aspect of this invention has a pH in a range of about 2.5-4.5, preferably about 2.8-3.5. Examples of buffers that will control the pH within this range include phosphate, acetate, citrate or ammonium buffers, or more than one. The preferred such buffers are citrate and ammonium buffers, most preferably ammonium sulfate or ammonium citrate buffers. The "loading buffer" is that which is used to load the mixture of the antibody and contaminant

on the HIC column and the "elution buffer" is that which is used to elute the antibody from the column. Often the loading buffer and elution buffer will be the same.

By "correctly disulfide linked" is meant that all cysteine residues in the antibody are covalently associated as disulfide bonds and these disulfide associations correspond to the disulfide associations of the native immunoglobulin. Circular dichroism as described in Example 1 may be used to determine whether or not an antibody is correctly disulfide linked by following the structural integrity of the molecule upon acid denaturation. An antibody is "incorrectly disulfide linked" when one or more cysteine residues are not covalently associated as disulfide bonds or are covalently associated with cysteine residues with which they are normally not associated in the native immunoglobulin.

10

Modes for Carrying Out the Invention

The process herein involves purifying an antibody from its related variants, usually after the antibody has already been purified from most other impurities. This purification step may be the final one before therapeutic formulation or it may be followed by other purification step(s). While the antibody in the mixture of variants may be produced from any source (e.g. peptic cleavage of intact antibodies), preferably it is made recombinantly. Techniques for production of antibodies, including antibody fragments, follow.

15

1. Antibody Preparation

(i) Polyclonal antibodies

Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

20

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining 1 mg or 1 μg of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

30

35

(ii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring

mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods (Cabilly *et al.*,
5 *supra*).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to
10 form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase
15 (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21
20 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 [1984]; Brodeur *et al.*, Monoclonal Antibody Production Techniques and Applications, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

25 Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard
30 analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *supra*). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

35 The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-SepharoseTM, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding

the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, Curr. Opinion in Immunol., 5:256-262 (1993) and Plückthun, Immunol. Revs., 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, Nature, 348:552-554 (1990), using the proper antigen such as CD11a, CD18, IgE, or HER-2 to select for a suitable antibody or antibody fragment. Clackson *et al.*, Nature, 352:624-628 (1991) and Marks *et al.*, J. Mol. Biol., 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Mark *et al.*, Bio/Technology, 10:779-783 [1992]), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, Nuc. Acids. Res., 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of "monoclonal" antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (Cabilly *et al.*, *supra*; Morrison, *et al.*, Proc. Nat. Acad. Sci., 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

For diagnostic applications, the variants herein derived from antibodies typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I ; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, e.g., ^{125}I , ^{32}P , ^{14}C , or ^3H ; or an enzyme, such as alkaline phosphatase, beta-galactosidase, or horseradish peroxidase.

Any method known in the art for separately conjugating the polypeptide variant to the detectable moiety may be employed, including those methods described by Hunter *et al.*, Nature, 144:945 (1962); David *et al.*, Biochemistry, 13:1014 (1974); Pain *et al.*, J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

(iii) Humanized and human antibodies

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, Nature, 321:522-525 [1986]; Riechmann *et al.*, Nature, 332:323-327 [1988]; Verhoeven *et al.*, Science, 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly *et al.*, *supra*), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al.*, J. Immunol., 151:2296 [1993]; Chothia and Lesk, J. Mol. Biol., 196:901 [1987]). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, Proc. Natl. Acad. Sci. USA, 89:4285 [1992]; Presta *et al.*, J. Immunol., 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*,

Jakobovits *et al.*, Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits *et al.*, Nature, 362:255-258 (1993); Bruggermann *et al.*, Year in Immuno., 7:33 (1993). Human antibodies can also be produced in phage-display libraries (Hoogenboom and Winter, J. Mol. Biol., 227:381 [1991]; Marks *et al.*, J. Mol. Biol., 222:581 [1991]).

5 (iv) Antibody fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto *et al.*, Journal of Biochemical and Biophysical Methods 24:107-117 [1992] and Brennan *et al.*, Science, 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody
10 fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')₂ fragments (Carter *et al.*, Bio/Technology 10:163-167 [1992]). Alternatively, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

15 (v) Bispecific antibodies

Bispecific antibodies (BsAbs) are antibodies that have binding specificities for at least two different antigens. Bispecific antibodies can be derived from full length antibodies or antibody fragments (e.g. F(ab')₂ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length
20 bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein and Cuello, Nature, 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather
25 cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker *et al.*, EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least
30 part of the hinge, C_H2, and C_H3 regions. It is preferred to have the first heavy-chain constant region (C_H1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of
35 the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published March 3, 1994. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, Methods in Enzymology, 121:210 (1986).

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, Science, 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab' -TNB derivatives is then reconverted to the Fab' -thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab' -TNB derivative to form the BsAb. The BsAbs produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab' -SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby *et al.*, J. Exp. Med., 175:217-225 (1992) describe the production of a fully humanized BsAb $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the BsAb. The BsAb thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets. See also Rodriguez *et al.*, Int. J. Cancers, (Suppl.) 7:45-50 (1992).

Various techniques for making and isolating BsAb fragments directly from recombinant cell culture have also been described. For example, bispecific $F(ab')_2$ heterodimers have been produced using leucine zippers. Kostelny *et al.*, J. Immunol., 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. The "diabody" technology described by Hollinger *et al.*, Proc. Natl. Acad. Sci. (USA), 90:6444-6448 (1993) has provided an alternative mechanism for making BsAb fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too

short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making BsAb fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, J. Immunol., 152:5368 (1994). These researchers
 5 designed an antibody which comprised the V_H and V_L domains of a first antibody joined by a 25-amino-acid-residue linker to the V_H and V_L domains of a second antibody. The refolded molecule bound to fluorescein and the T- cell receptor and redirected the lysis of human tumor cells that had fluorescein covalently linked to their surface.

10 2. Antibody Purification

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter *et al.*, Bio/Technology 10:163-167 (1992) describe a procedure for isolating antibodies
 15 which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be
 20 included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells is preferably subjected to at least one purification step prior to LPHIC. Examples of suitable purification steps include hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred
 25 purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark *et al.*, J. Immunol. Meth. 62:1-13 [1983]). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss *et al.*, EMBO J. 5:1567-1575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available.
 30 Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABXTM resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SepharoseTM, chromatography
 35 on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminant(s) is subjected to LPHIC. Often, the antibody composition to be purified will be present in a buffer from the previous purification step. However, it may be necessary to add a buffer to the antibody

composition prior to the LPHIC step. Many buffers are available and can be selected by routine experimentation. The pH of the mixture comprising the antibody to be purified and at least one contaminant in a loading buffer is adjusted to a pH of about 2.5-4.5 using either an acid or base, depending on the starting pH. Preferably, the loading buffer has a low salt concentration (*i.e.* less than about 0.25M salt).

5 The mixture is loaded on the HIC column. HIC columns normally comprise a base matrix (*e.g.* cross-linked agarose or synthetic copolymer material) to which hydrobobic ligands (*e.g.* alkyl or aryl groups) are coupled. The preferred HIC column comprises an agarose resin substituted with phenyl groups (*e.g.* a Phenyl SepharoseTM column). Many HIC columns are available commercially. Examples include, but are not limited to, Phenyl SepharoseTM 6 Fast Flow column with low or high substitution (Pharmacia LKB Biotechnology, AB, Sweden); Phenyl SepharoseTM High Performance column (Pharmacia LKB Biotechnology, AB, Sweden); Octyl SepharoseTM High Performance column (Pharmacia LKB Biotechnology, AB, Sweden); FractogelTM EMD Propyl or FractogelTM EMD Phenyl columns (E. Merck, Germany); Macro-PrepTM Methyl or Macro-PrepTM *t*-Butyl Supports (Bio-Rad, California); WP HI-Propyl (C₃)TM column (J.T. Baker, New Jersey); and ToyopearlTM ether, phenyl or butyl columns (TosoHaas, PA).

15 The antibody is eluted from the column using an elution buffer which is normally the same as the loading buffer. The elution buffer can be selected using routine experimentation. The pH of the elution buffer is between about 2.5-4.5 and has a low salt concentration (*i.e.* less than about 0.25M salt). It has been discovered that it is not necessary to use a salt gradient to elute the antibody of interest; the desired product is recovered in the flow through fraction which does not bind significantly to the column.

20 The LPHIC step provides a way to remove a correctly folded and disulfide bonded antibody from unwanted contaminants (*e.g.* incorrectly associated light and heavy fragments). In particular, the method provides a means to substantially remove an impurity characterized herein as a correctly folded antibody fragment whose light and heavy chains fail to associate through disulfide bonding. It has been discovered that the antibody composition prepared using the LPHIC described herein is at least 95% pure. Purities of more than 98% have been achieved using the method described in Example 1.

25 The antibody composition prepared by LPHIC can be further purified as desired using techniques which are well known in the art. Diagnostic or therapeutic formulations of the purified protein can be made by providing the antibody composition in a physiologically acceptable carrier, examples of which are provided below.

30 To remove contaminants (*e.g.* unfolded antibody and incorrectly associated light and heavy fragments) from the HIC column so that it can be re-used, a composition including urea (*e.g.* 6.0 M urea, 1% MES buffer pH 6.0, 4mM ammonium sulfate) can be flowed through the column.

3. Uses for the Purified Antibody

35 Many uses for antibodies which have been purified using the disclosed method are contemplated, including diagnostic and therapeutic uses. Various diagnostic and therapeutic uses for antibodies have been reviewed in Goldenberg *et al.*, Semin. Cancer Biol. 1(3):217-225 (1990), Beck *et al.*, Semin. Cancer Biol. 1(3):181-188 (1990), Niman, Immunol. Ser. 53:189-204 (1990) and Endo, Nippon Igaku Hoshasen Gakkai Zasshi (Japan) 50(8):901-909 (1990), for example.

The antibodies described herein can be used in immunoassays, such as enzyme immunoassays. BsAbs are particularly useful for this type of assay: one arm of the BsAb can be designed to bind to a specific epitope on the enzyme so that binding does not cause enzyme inhibition, the other arm of the antibody can be designed to bind to an immobilizing matrix ensuring a high enzyme density at the desired site. Examples of such diagnostic BsAbs include those having specificity for IgG as well as ferritin, and those having binding specificities for horseradish peroxidase (HRP) as well as a hormone, for example.

The antibodies can be designed for use in two-site immunoassays. For example, two antibodies are produced binding to two separate epitopes on the analyte protein; one antibody binds the complex to an insoluble matrix, the other binds an indicator enzyme.

Antibodies can also be used for *in vitro* or *in vivo* immunodiagnosis of various diseases such as cancer. To facilitate this diagnostic use, an antibody which binds a tumor associated antigen can be conjugated with a detectable marker (e.g. a chelator which binds a radionuclide). For example, a antibody having specificity for the tumor associated antigen CEA can be used for imaging of colorectal and thyroid carcinomas. The anti-p185^{HER2} antibody disclosed herein can be used for detecting cancers characterized by amplification of the HER2 protooncogene. Other non-therapeutic, diagnostic uses for the antibody will be apparent to the skilled practitioner.

For diagnostic applications, the antibody typically will be labeled directly or indirectly with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ³H, ¹⁴C, ³²P, ³⁵S, or ¹²⁵I; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; or an enzyme, such as alkaline phosphatase, beta-galactosidase or HRP.

Any method known in the art for separately conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter *et al.*, Nature 144:945 (1962); David *et al.*, Biochemistry 13:1014 (1974); Pain *et al.*, J. Immunol. Meth. 40:219 (1981); and Nygren, J. Histochem. and Cytochem. 30:407 (1982).

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of analyte in the test sample is inversely proportional to the amount of standard that becomes bound to the antibody. To facilitate determining the amount of standard that becomes bound, the antibody generally is insolubilized before or after the competition, so that the standard and analyte that are bound to the antibody may conveniently be separated from the standard and analyte which remain unbound.

BsAbs are particularly useful for sandwich assays which involve the use of two molecules, each capable of binding to a different immunogenic portion, or epitope, of the sample to be detected. In a sandwich assay, the test sample analyte is bound by a first arm of the antibody which is immobilized on a solid support, and thereafter a second arm of the antibody binds to the analyte, thus forming an insoluble three part complex. See, e.g., US Pat No. 4,376,110. The second arm of the antibody may itself be labeled with a detectable moiety

(direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

5 The antibodies also are useful for the affinity purification of an antigen of interest from recombinant cell culture or natural sources.

Therapeutic uses for the antibodies purified using the method described herein are also contemplated. For example, the antibody can be used for redirected cytotoxicity (e.g. to kill tumor cells), as a vaccine adjuvant, for delivering thrombolytic agents to clots, for delivering immunotoxins to tumor cells, for converting enzyme activated prodrugs at a target site (e.g. a tumor), for treating infectious diseases or targeting immune
10 complexes to cell surface receptors. Therapeutic formulations of the antibody are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., [1980]), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other
15 organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic
20 surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions.
25 Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

The antibody to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The antibody ordinarily will be stored in lyophilized form or in solution.

Therapeutic antibody compositions generally are placed into a container having a sterile access port,
30 for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of antibody administration is in accord with known methods, e.g., injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems as noted below. The antibody is administered continuously by infusion or by bolus injection.

35 Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels [e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, J. Biomed. Mater. Res. 15:167-277 (1981) and Langer, Chem. Tech. 12:98-105 (1982) or poly(vinylalcohol)], polylactides (U.S. Patent No. 3,773,919, EP

58.481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, Biopolymers 22:547-556 [1983]), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron DepotTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133.988).

5 While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for antibody stabilization depending on the mechanism involved. For example, if the
10 aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release antibody compositions also include liposomally entrapped antibody. Liposomes
15 containing the antibody are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. USA 82:3688-3692 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. USA 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected
20 proportion being adjusted for the optimal antibody therapy.

An effective amount of antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 µg/kg to up to 10 mg/kg or more,
25 depending on the factors mentioned above. Typically, the clinician will administer antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

30

EXAMPLE 1

LOW pH HYDROPHOBIC INTERACTION CHROMATOGRAPHY (LPHIC)

A method to preferentially unfold non disulfide linked antibody by acid denaturation has been developed. During acid denaturation, intermolecular charge repulsion contributes to unfolding and the extent
35 of unfolding depends on the conditions of acidification as well as protein structure. At low pH, unfolded antibody and incorrectly associated light and heavy fragments can be separated by LPHIC (flow through mode). Unwanted antibody species bind to the column while desired antibody fragments flow through. Impurities can be removed from the column with 6.0 M urea, 1% MES buffer (pH 6.0), 4mM ammonium sulfate.

The following antibodies were subjected to LPHIC:

- (a) humanized anti-CD18 Fab' and F(ab')₂;
- (b) chimeric anti-CD18 Fab' and F(ab')₂;
- (c) linear humanized anti-CD18 F(ab')₂; and
- (d) linear humanized anti-p185^{HER2} F(ab')₂.

5

MATERIALS AND METHODS

Cell Material. Transformed *E. coli* strains were used to produce humanized anti-CD18 Fab' H52, version OZ (huMAb H52OZG1) as described in Eigenbrot *et al.*, Proteins: Structure, Function and Genetics, 18:49-62 (1994). A chimeric version of anti-CD18 MAb, MHM23 (Hildreth *et al.*, Eur. J. Immunol. 13:202-208 [1983]), was prepared having the light chain sequence SEQ ID No. 1 and heavy chain sequence SEQ ID No. 2. The sequences encoding the Fab were subcloned into a vector based upon pAK19 which has previously been described by Carter *et al.*, Bio/Technology 10:163-167 (1992). Linear humanized anti-CD18 huMAbH52 and anti-p185^{HER2} huAb4D5-8 F(ab')₂ fragments were produced as described in Example 2 below.

Reverse Phase Chromatography Analysis. Reverse Phase chromatography was carried out on a Reverse Phase PLRP-STM 4.6 x 50mm column, 8mm particle size, (Polymer Laboratories, Shropshire, UK) maintained at 50°C. The proteins were eluted using an increasing linear gradient from 31% Buffer B to 41% Buffer B. Buffer A contained 0.1% trifluoroacetic acid in deionized water, and Buffer B contained 0.1% trifluoroacetic acid in HPLC grade acetonitrile. The flow rate was maintained at 2ml/min, and the detection wavelength was 214 nm.

Extraction of Fab' antibody fragments from *E. coli* and protection of free sulfhydryl with 4,4-DTP. Antibody fragments were extracted from *E. coli* frozen cell pellets obtained from 10 liter fermentations. Since the cells were completely disrupted, 4,4-dithiodipyridine (4,4-DTP) was added to protect the free cysteine in Fab' antibody fragment engineered to contain a free thiol in the hinge region (anti-CD18 huMAb H52OZG1 and anti-CD18 MAb MHM23). Linear F(ab')₂ versions without engineered free cysteines in the hinge region (anti-CD18 huMAbH52 and huMAb4D5-8 linear versions) were extracted without 4,4-DTP as described in Example 2 below.

Extraction. Frozen cell pellets were re-suspended at room temperature in 20mM MES buffer, pH 6.0 containing 5 mM EDTA and 20 mM 4,4'-DTP previously dissolved in ethanol (3 liters of buffer/kg of cell pellet). The suspended cells were disrupted by two passages through a Mantin Gaulin homogenizer at 5500 to 6500 PSI. The homogenate was adjusted to 0.25% (v/v) with polyethyleneimine (PEI) and diluted with an equal volume of 2-8°C purified water. The diluted homogenate was then centrifuged. The antibody fragment was found in the supernatant.

Purification of the protected Fab'-TP antibody fragments. ABXTM chromatography was used for the initial purification of the antibody fragments from *E. coli* proteins. To further purify the antibody fragments from antibody species that lack a disulfide bond between the light and heavy chains, a low pH hydrophobic interaction chromatography step was introduced.

ABXTM chromatography. The supernatant containing the antibody fragment was diluted to a conductivity of 2 millisiemens or less with purified water. The diluted supernatant was pumped sequentially through 0.5 and 0.22 micron filters and loaded onto a ABXTM column (J. T. Baker Phillipsburg, NJ)

equilibrated in 50 mM MES/5mM EDTA, pH 6.0 (Buffer A). The effluent was monitored at 280 nm. After loading, the column was washed with Buffer A for 2 column volumes. Antibodies were eluted with a 20 column volume gradient from 0 to 50 mM ammonium sulfate in Buffer A. Fractions were analyzed by HPLC and pooled accordingly.

5 **Low pH Hydrophobic Interaction Chromatography (LPHIC).** The ABXTM purified Fab' pools (humanized and chimeric anti-CD18) were adjusted to 20mM NaPO₄ and the pH of the pools was adjusted to 3.1 using 6N HCL immediately prior to loading on a Phenyl SepharoseTM Fast Flow (Pharmacia Biotech Inc. Piscataway, NJ) column. Chemically coupled F(ab')₂ (humanized and chimeric anti-CD18) and linear F(ab')₂ (anti-CD18 and anti-p185^{HER2}) ABXTM pools were prepared in the same way except that they were made
10 20mM in ammonium sulfate. A typical flow through chromatogram of the LPHIC is shown in Fig. 1. The pH of the LPHIC purified antibody was immediately adjusted to pH 5, with 10 % NaOH.

pH analysis. An experiment was designed to determine the pH at which maximum purification as well as maximum yield could be achieved. ABXTM pools were made 25mM in NaPO₄ and the pH was adjusted using 6N HCl. After the desired pH was obtained, the samples were flowed through a Phenyl
15 SepharoseTM Fast Flow column and the pools were analyzed using Reverse Phase HPLC to determine purity and yield.

Circular Dichroism. Spectra was recorded on an AVIV model 60DS instrument at 25°C. Path length cells of 1mm were used for far UV measurements and 10 mm path length cells for near UV measurements. The rhuMAb H52OZG1 and rhuMAb H52OZG2 purified antibody samples were buffer exchanged into 10mM
20 KPO₄ buffer by gel permeation chromatography on Sephadex G25TM (Pharmacia Biotech Inc. Piscataway, NJ). The samples were titrated with phosphoric acid to desired pH prior to measuring the CD spectra.

RESULTS AND DISCUSSION

CD Spectroscopy of rhuMAb H52OZG1 and rhuMAb H52OZG2. The ABXTM purified pool
25 appears to contain small amounts of antibody fragments whose light and heavy chains are correctly folded but fail to covalently associate through a disulfide bond. This impurity can be detected on SDS gels and by analytical Reverse Phase HPLC (Fig. 2). The non-covalently associated antibody fragments can be separated from the desired product by preferential acid denaturation followed by LPHIC. To determine the acid denaturation differences between the disulfide associated and non disulfide associated species two purified
30 antibody fragments were used; rhuMAb H52OZG2 and rhuMAb H52OZG1. RhuMAb H52OZG2 is a mutant of rhuMAb H52OZG1 in which the cysteine residues 215 and 228 in the light and the heavy chains respectively have been changed to serine residues. This mutant should mimic the acid denaturation behavior of non disulfide linked antibodies. Near-UV and far-UV spectra of rhuMAb H52OZG2 and rhuMAb H52OZG1 at
different pH values show different denaturation transition points (Figs. 3A-3D). A transition point represents
35 a change from correctly folded antibody fragment, to its unfolded state. Non disulfide associated fragments can be denatured around pH 3.2, whereas the disulfide associated fragments required pH values below 2.5 for denaturation.

LPHIC pH analysis. From the acid denaturation analysis it can be concluded that around pH 3.0 the non disulfide associated fragments can be denatured and then preferentially bound to a Phenyl SepharoseTM

Fast Flow column. In order to evaluate the effect of varying low pH's on the LPHIC step, ABXTM purified antibody pools were LPHIC purified at different pH values. Analysis of the purified pools was performed using Reverse Phase HPLC. A bar graph was generated in which purity, yield, and light chain percentages were determined from the LPHIC purifications (Fig. 4). From the bar graph it was determined that pH 3.1 was the best value for balancing purity and yield for the purification of rhuMAb H52OZG1 antibody. Large scale purifications of ABXTM pools were carried out at pH 3.1.

SUMMARY

LPHIC made possible the purification of Fab', L-F(ab')₂ and chemically linked F(ab')₂ antibody fragments from unwanted antibody species to more than 98% purity. Flow of samples through a Phenyl SepharoseTM Fast Flow column at low pH removed antibodies that lack disulfide bonds between heavy and light chains as well as incorrectly associated light and heavy chain species. Circular Dichroism studies of disulfide linked and non-disulfide linked anti-CD18 F(ab')₂ antibodies (rhuMAb H52OZG1 and rhuMAb H52OZG2) demonstrated that the non disulfide linked antibody (rhuMAb H52OZG2) denatured into light and heavy chain molecules at pH 3.2. Disulfide linked antibody (rhuMAb H52OZG1), denatured at pH 2.5. Chromatography experiments at different pH values demonstrated that pH 3.1 represents the best value for balancing purity and yield for purification of anti-CD18 rhuMAb H52OZG1.

EXAMPLE 2

20 LINEAR ANTIBODY PRODUCTION

This example describes the production of bivalent, linear (L-)F(ab')₂ fragments (comprising tandem repeats of a heavy chain fragment, V_H-C_H1-V_H-C_H1 cosecreted with a light chain) which were subjected to LPHIC (see Example 1 above).

25 MATERIALS AND METHODS

Construction of L-F(ab')₂ variants. The expression plasmid, pAK19, for secretion of huMAb4D5-8 Fab' fragment has previously been described (Carter *et al.*, Bio/Technology 10:163-167 [1992]). Plasmids pLA1, pLA2 and pLA3 were designed to secrete L-F(ab')₂ variants v1, v2 and v3, respectively (Fig. 5A). Plasmid pLA1 was constructed from pAK19 by modifying the heavy chain to encode tandem huMAb4D5-8 Fd segments: V_H-C_H1-V_H-C_H1. L-F(ab')₂ v2 and v3 were constructed from pLA1 by precisely replacing 5' or 3' copies of V_H in pLA1, respectively, with that from the humanized anti-CD18 Ab, huMAb H52OZ (Eigenbrot *et al.*, *supra*). A plasmid was designed to secrete L-F(ab')₂ anti-CD18. A plasmid was constructed from anti-CD18 Ab, huMAb H52OZ (Eigenbrot *et al.*, *supra*) by modifying the heavy chain to encode tandem Fd segments V_H-C_H1-V_H-C_H1.

35 *E. coli* expression and purification of L-F(ab')₂ variants. The production of huMAb4D5-8 Fab and thioether-linked F(ab')₂ fragments from *E. coli* has previously been described by Kelley *et al.*, Biochemistry 31:5434-5441 (1992) and Rodriguez *et al.*, J. Immunol. 151:6954-6961 (1993). L-F(ab')₂ variants were secreted from *E. coli* strain 33B6 (Rodriguez *et al.*, Cancer Res. 55:63-70 ([1995]) containing corresponding expression plasmids grown for 40h at 30°C in an aerated 10 liter fermentor as previously

described (Carter *et al.*, *supra*). Expression titers were estimated by antigen (Ag)-binding ELISA (Carter *et al.*, *supra*). L-F(ab')₂ variants were purified from 400g of corresponding fermentation pastes thawed in the presence of 2 liters 20mM MES, 5 mM EDTA, pH 6.0 (ME buffer). Resuspended cells were disrupted by three passages through a microfluidizer (Microfluidics Corporation, Newton, MA) and adjusted to 0.25 % (v/v) polyethyleneimine. Solid debris was removed by centrifugation (7,300g, 30 min, 4°C). The supernatant was diluted with an equal volume of distilled water and then loaded onto a 20 ml Bakerbond ABXTM column (J. T. Baker, Phillipsburg, NJ) pre-equilibrated with ME buffer. L-F(ab')₂ was eluted using a linear gradient of 0-50 mM (NH₄)₂SO₄ in ME buffer. Pooled L-F(ab')₂ was adjusted to 25 mM Na₂HPO₄, pH 3.0 and passed over a 20 ml Phenyl SepharoseTM Fast Flow column (high sub) (Pharmacia, Piscataway, NJ) equilibrated with 25 mM Na₂HPO₄, 20 mM (NH₄)₂SO₄, pH 3.0. The flow through fractions containing L-F(ab')₂ were pooled and adjusted to pH 6.0.

All antibody fragments were buffer-exchanged into PBS by S100-HRTM (Pharmacia) size exclusion chromatography (2.5 cm x 100 cm). Residual endotoxin was removed by repeated passage through PyroBind-STTM filters (Sepracor, Marlborough, MA). The endotoxin level of each preparation was estimated by the limulus amebocyte lysate test (Associates of Cape Cod Inc., Woods Hole, MA). The purified antibody (Ab) fragments were passed through a 0.2mm filter, flash-frozen in liquid nitrogen, and stored at -70°C until required.

Analysis of Ab fragment binding to p185^{HER2} ECD. The affinity and kinetics of binding of huMAb4D5-8 Ab fragments to p185^{HER2} ECD (Fendly *et al.*, *J. Biol. Resp. Mod.* 9:449-455 [1990]) were determined by surface plasmon resonance using the BIAcore system (Pharmacia) as previously described by Kelley and O'Connell in *Biochemistry*, 32:6828-6835 (1993). The stoichiometry of binding of Ab fragments to Ag was determined in solution. Briefly, varying amounts of p185^{HER2} ECD (Fendly *et al.*, *supra*) in PBS were added to a fixed quantity of Ab fragment (15-20 mg) and the mixture then analyzed by size exclusion FPLC using a Superose 12TM column (Pharmacia) equilibrated with 0.1M NaH₂HPO₄, pH 6.7.

Cell proliferation assay. The effect of huMAb4D5-8 Ab fragments (0-30 mg/ml) upon the proliferation of the human mammary adenocarcinoma cell line, BT474, was investigated as previously described (Hudziak *et al.*, *Molec. Cell. Biol.* 9:1165-1172 [1989]).

Pharmacokinetics of Ab fragments in normal mice. Groups of female CD-1 mice (20-32 g, n = 45) from Hanlen Sprague Dawley (Indianapolis, IN) received one of the huMAb4D5-8 Ab fragments (3-4 mg/ml in PBS) by rapid tail vein injection (10 mg/kg). Three mice per time point were sacrificed at scheduled times ranging from 1 min to 24 h post injection, and samples of their serum were collected and stored frozen. Serum concentrations of each Ab fragment were determined by Ag-binding ELISA as previously described using corresponding fragments as standards (Rodriguez *et al.*, *supra*). Only the thioether-linked F(ab')₂ was detectable (0.7 mg/ml) at 24 h following injection.

Data from each treatment group were analyzed by fitting a biexponential function, $C(t) = Ae^{-at} + Be^{-bt}$, to the mean values of the serum concentrations at each time point. Exponential components were estimated by a non-linear least squares method using the Gauss-Newton-Marquardt-Levenberg procedure (Press *et al.*, *Numerical Recipes in C*, Cambridge University Press, Cambridge, UK, [1988]) and a weighing of y^{-2} , where y is the measured serum concentration. Initial volume of distribution (V_1), volume of distribution at

steady state (V_{ss}), clearance time (CL), plus initial and terminal half-lives ($t_{1/2}$) were then calculated from the estimated parameters as described (Wagner, J. Pharmacokin. Biopharm. 4:443-467 [1976]):

$$C_0 = A + B$$

$$V_1 = \text{Dose} / C_0$$

$$V_{ss} = (A / a^2 + B / b^2) / (AUC)^2$$

$$CL = \text{Dose} / AUC$$

$$\text{Initial } t_{1/2} = \ln 2 / a$$

$$\text{Terminal } t_{1/2} = \ln 2 / b$$

where C_0 is the extrapolated initial concentration, and AUC is the area under the fitted plasma concentration *versus* time curve. The permanence time (T), which is the expected interval of time spent by the drug in all its passages through a compartment (in this case, serum) was estimated (Mordenti and Rescigno, Pharm. Res., 9:17-25 [1992]) as follows:

$$T = AUC / C_0.$$

RESULTS AND DISCUSSION

Design of L-F(ab')₂. L-F(ab')₂ variants were designed to comprise a heavy (H) chain of tandem Fd fragments, V_H-C_H1-V_H-C_H1, associated with two copies of the corresponding light (L) chain (Fig. 5A). At the Fd-Fd junction, the C-terminus of C_H1 (... THT) is joined directly to the N-terminus of V_H (EVQ ...) without any extraneous linking protein sequences. This was an attempt to minimize the potential risks of immunogenicity and susceptibility to serum proteases in patients. A potential drawback of this strategy of omitting linkers is that accessibility of the C-terminal binding site to antigen (Ag) might be impaired.

The huMAb4D5-8 L-F(ab')₂ variant, v1, was designed to have two functional binding sites for the Ag, p185^{HER2} ECD (Fig. 5A). In contrast, huMAb4D5-8 L-F(ab')₂ v2 and v3 were designed to have a single Ag binding site. This was accomplished by replacing either 5' or 3' copies of V_H in L-F(ab')₂ v1 with that from the anti-CD18 Ab, huMAbH52 OZ (Eigenbrot *et al.*, *supra*). A Fab comprising the L chain from huMAb4D5-8 and the H chain Fd fragment from huMAbH52 OZ was expressed and purified and found to not bind p185^{HER2} ECD as anticipated.

Production and *in vitro* characterization of antibody (Ab) fragments. L-F(ab')₂ variants were expressed in *E. coli* by cosecretion of the L chain with the tandem H chain Fd fragments from a dicistronic operon (Fig. 5B). Titers of ≥ 100 mg/l functional (Ag-binding) huMAb4D5-8 L-F(ab')₂ were achieved following culturing of *E. coli* containing corresponding expression plasmids to high cell density in the fermentor. L-F(ab')₂ were recovered directly from *E. coli* by fully disrupting corresponding fermentation pastes followed by ABXTM, low pH hydrophobic interaction, and size exclusion chromatography. The endotoxin concentration was estimated as ≤ 0.32 endotoxin units per mg of purified protein.

Purified huMAb4D5-8 L-F(ab')₂ variants together with F(ab')₂ and Fab were analyzed by SDS-PAGE. Under non-reducing conditions the three huMAb4D5-8 L-F(ab')₂ variants (M_r ~96 kDa) and the Fab fragment (M_r ~48 kDa) show one major band with the anticipated electrophoretic mobility. The thioether-linked F(ab')₂ fragment (M_r ~96 kDa) shows anomalously retarded mobility as compared to the 97 kDa standard.

Under reducing conditions all huMAb4D5-8 Ab fragments give rise to a band of ~23 kDa apparent molecular weight anticipated for free L chain. In addition, L-F(ab')₂ and F(ab')₂ give rise to a band of ~48 kDa as anticipated from the presence of tandem and thioether-linked H chain dimers, respectively. The reduced H and L chains for the Fab fragment are not resolved under the electrophoretic conditions used.

- 5 Analysis of binding of Ab fragments to p185^{HER2} ECD. The stoichiometry of the Ab-Ag interaction was investigated by titration of huMAb4D5-8 Ab fragments with p185^{HER2} ECD followed by size exclusion chromatographic analysis (Figs. 6A-6C). huMAb4D5-8 L-F(ab')₂ v1 and F(ab')₂ show very similar titration profiles with p185^{HER2} ECD and bind two equivalents of antigen (Figs. 6A and 6B). As anticipated, the Fab fragment binds one equivalent of Ag (Fig. 6C). L-F(ab')₂ v2 and v3 bind only a single equivalent of
- 10 Ag.

The affinity and kinetics of binding of huMAb4D5-8 Ab fragments to Ag were investigated by surface plasmon resonance using immobilized p185^{HER2} ECD. See Table 1 below.

Table 1

- 15 Binding analysis of anti-p185^{HER2} Ab fragments to p185^{HER2} ECD^a

Ab Fragment	k_{on}^b s ⁻¹ M ⁻¹	k_{off}^b s ⁻¹	K_d nM
Fab	3.4×10^5	5.2×10^{-4}	1.5
20 L-F(ab') ₂ v1	6.4×10^5	2.9×10^{-4}	0.46
L-F(ab') ₂ v2	2.3×10^5	2.8×10^{-4}	1.2
L-F(ab') ₂ v3	5.7×10^5	3.1×10^{-5}	5.5
F(ab') ₂	1.9×10^6	3.2×10^{-4}	0.17

- 25 ^a Data obtained by surface plasmon resonance.

^b SE of estimates $\pm 3\%$

- The bivalent L-F(ab')₂ variant, v1, binds Ag with 3-fold lower affinity than the does F(ab')₂. This mainly reflects a small decrease in association rate between F(ab')₂ and L-F(ab')₂, respectively. The monovalent L-F(ab')₂ variants v2 and v3 show approximately 3-fold and 12-fold weaker binding than the
- 30 bivalent L-F(ab')₂ variant, v1. Thus both binding sites in the L-F(ab')₂ are competent for binding p185^{HER2} ECD, although the efficiency of Ag binding is apparently slightly impaired for the C-terminal site. As anticipated, the binding affinity of L-F(ab')₂ v2 is very similar to the corresponding Fab fragment.

- Antiproliferative activity of Ab fragments.** The antiproliferative activity of huMAb4D5-8 Ab fragments was investigated using the p185^{HER2}-overexpressing breast tumor cell line, BT474 (see Fig. 7).
- 35 Proliferation of BT474 in the presence of saturating quantities of L-F(ab')₂ v1 and thioether-linked F(ab')₂ are approximately 40% and 55% of the untreated control, respectively. Thus L-F(ab')₂ v1 is more potent in blocking the proliferation of BT474 cells than is the thioether-linked F(ab')₂ fragment. The antiproliferative

activities of the monovalent L-F(ab')₂ variants, v2 and v3, approach that of the bivalent L-F(ab')₂ variant, v1, and are much greater than for the Fab fragment.

Pharmacokinetic characterization of Ab fragments in normal mice. The time course of huMAb4D5-8 Fab, F(ab')₂ and L-F(ab')₂ variants in the serum of normal mice was determined from serial sacrifice of multiple animals (Fig. 8) and used for calculation of pharmacokinetic parameters. See Table 2 below.

Table 2
Pharmacokinetic parameters for anti-p185^{HER2} Ab fragments
in normal mice^a

10

Ab Fragment	V ₁ ml kg ⁻¹	V _{ss} ml kg ⁻¹	CL h ⁻¹ kg ⁻¹	t _{1/2} hours		T hours
				initial	terminal ^b	
L-F(ab') ₂ v1	29.0	38.0	19.3	0.40	1.6	1.50
F(ab') ₂	36.1	44.0	20.6	0.99	2.6	1.75
15 Fab	22.6	35.4	106	0.14	1.6	0.21

^a Pharmacokinetic parameters were calculated from the data in Figure 3.

20 ^b The terminal half-life contributes 83%, 30% and 6.5% to the total AUC for L-F(ab')₂ v1, F(ab')₂ and Fab, respectively.

L-F(ab')₂ and thioether-linked F(ab')₂ are very similar in terms of their pharmacokinetic parameters, while the Fab fragment is cleared more rapidly. The permanence times in serum for L-F(ab')₂ v1 and thioether-linked F(ab')₂ fragments, are 7-fold and 8-fold greater than for the Fab fragment, respectively.

25

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT: Genentech, Inc.

(ii) TITLE OF INVENTION: ANTIBODY PURIFICATION

10 (iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Genentech, Inc.

(B) STREET: 460 Point San Bruno Blvd

15 (C) CITY: South San Francisco

(D) STATE: California

(E) COUNTRY: USA

(F) ZIP: 94080

20 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: WinPatin (Genentech)

25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 20-Apr-1995

(C) CLASSIFICATION:

30

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lee, Wendy M.

(B) REGISTRATION NUMBER: 00,000

(C) REFERENCE/DOCKET NUMBER: P0941PCT

35

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 415/225-1994

(B) TELEFAX: 415/952-9881

(C) TELEX: 910/371-7168

40

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 214 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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	1				5						10					15
10	Gly	Asp	Arg	Val	Thr	Ile	Asn	Cys	Arg	Ala	Ser	Gln	Asp	Ile	Asn	
					20					25						30
	Asn	Tyr	Leu	Asn	Trp	Tyr	Gln	Gln	Lys	Pro	Asn	Gly	Thr	Val	Lys	
					35					40						45
15	Leu	Leu	Ile	Tyr	Tyr	Thr	Ser	Thr	Leu	His	Ser	Gly	Val	Pro	Ser	
					50					55						60
	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Tyr	Ser	Leu	Thr	Ile	
20					65					70						75
	Ser	Asn	Leu	Asp	Gln	Glu	Asp	Ile	Ala	Thr	Tyr	Phe	Cys	Gln	Gln	
					80					85						90
25	Gly	Asn	Thr	Leu	Pro	Pro	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	
					95					100						105
	Ile	Arg	Arg	Ala	Val	Ala	Ala	Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro	
					110					115						120
30	Ser	Asp	Glu	Gln	Leu	Lys	Ser	Gly	Thr	Ala	Ser	Val	Val	Cys	Leu	
					125					130						135
	Leu	Asn	Asn	Phe	Tyr	Pro	Arg	Glu	Ala	Lys	Val	Gln	Trp	Lys	Val	
35					140					145						150
	Asp	Asn	Ala	Leu	Gln	Ser	Gly	Asn	Ser	Gln	Glu	Ser	Val	Thr	Glu	
					155					160						165
40	Gln	Asp	Ser	Lys	Asp	Ser	Thr	Tyr	Ser	Leu	Ser	Ser	Thr	Leu	Thr	
					170					175						180
	Leu	Ser	Lys	Ala	Asp	Tyr	Glu	Lys	His	Lys	Val	Tyr	Ala	Cys	Glu	

185 190 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn

200 205 210

5

Arg Gly Glu Cys

214

(2) INFORMATION FOR SEQ ID NO:2:

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 232 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1 5 10 15

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Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr

20 25 30

Glu Tyr Thr Met His Trp Met Lys Gln Ser His Gly Lys Ser Leu

25 35 40 45

Glu Trp Ile Gly Gly Phe Asn Pro Lys Asn Gly Gly Ser Ser His

50 55 60

30

Asn Gln Arg Phe Met Asp Lys Ala Thr Leu Ala Val Asp Lys Ser

65 70 75

Thr Ser Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Glu Asp

80 85 90

35

Ser Gly Ile Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly

95 100 105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val

40 110 115 120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu

125 130 135

	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu	Gly
					140					145					150
5	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp
					155					160					165
	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val
					170					175					180
10	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val
					185					190					195
	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn
					200					205					210
15	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys
					215					220					225
	Ser	Cys	Asp	Lys	Thr	His	Thr								
20					230		232								

CLAIMS

1. A process for purifying an antibody comprising loading a mixture containing the antibody on a hydrophobic interaction chromatography column and eluting the antibody from the column with a buffer having a pH of about 2.5-4.5.
2. The process of claim 1 wherein the mixture loaded onto the column is at a pH of about 2.5-4.5.
3. The process of claim 1 wherein the mixture loaded onto the column has a salt concentration of about 0-0.25M.
4. The process of claim 3 wherein the mixture loaded onto the column has a salt concentration of about 0-0.1M.
5. The process of claim 1 wherein the buffer has a salt concentration of about 0-0.25M.
6. The process of claim 5 wherein the buffer has a salt concentration of about 0-0.1M.
7. The process of claim 1 wherein the antibody is chimeric.
8. The process of claim 7 wherein the antibody is humanized.
9. The process of claim 1 wherein the antibody comprises an antibody fragment.
10. The process of claim 9 wherein the antibody fragment comprises a $F(ab')_2$ fragment.
11. The process of claim 1 wherein the buffer has a pH of about 2.8-3.5.
12. The process of claim 11 wherein the buffer has a pH of about 3.1.
13. The process of claim 1 wherein the hydrophobic interaction chromatography column is a phenyl agarose column.
14. The process of claim 1 wherein the purified antibody is correctly disulfide linked.
15. The process of claim 1 wherein the antibody is purified from an incorrectly disulfide linked antibody.
16. The process of claim 15 wherein the incorrectly disulfide linked antibody is an antibody fragment.
17. A composition comprising an antibody prepared by the process of claim 1 in a physiologically acceptable carrier.
18. The composition of claim 17 wherein the composition comprises an antibody fragment.

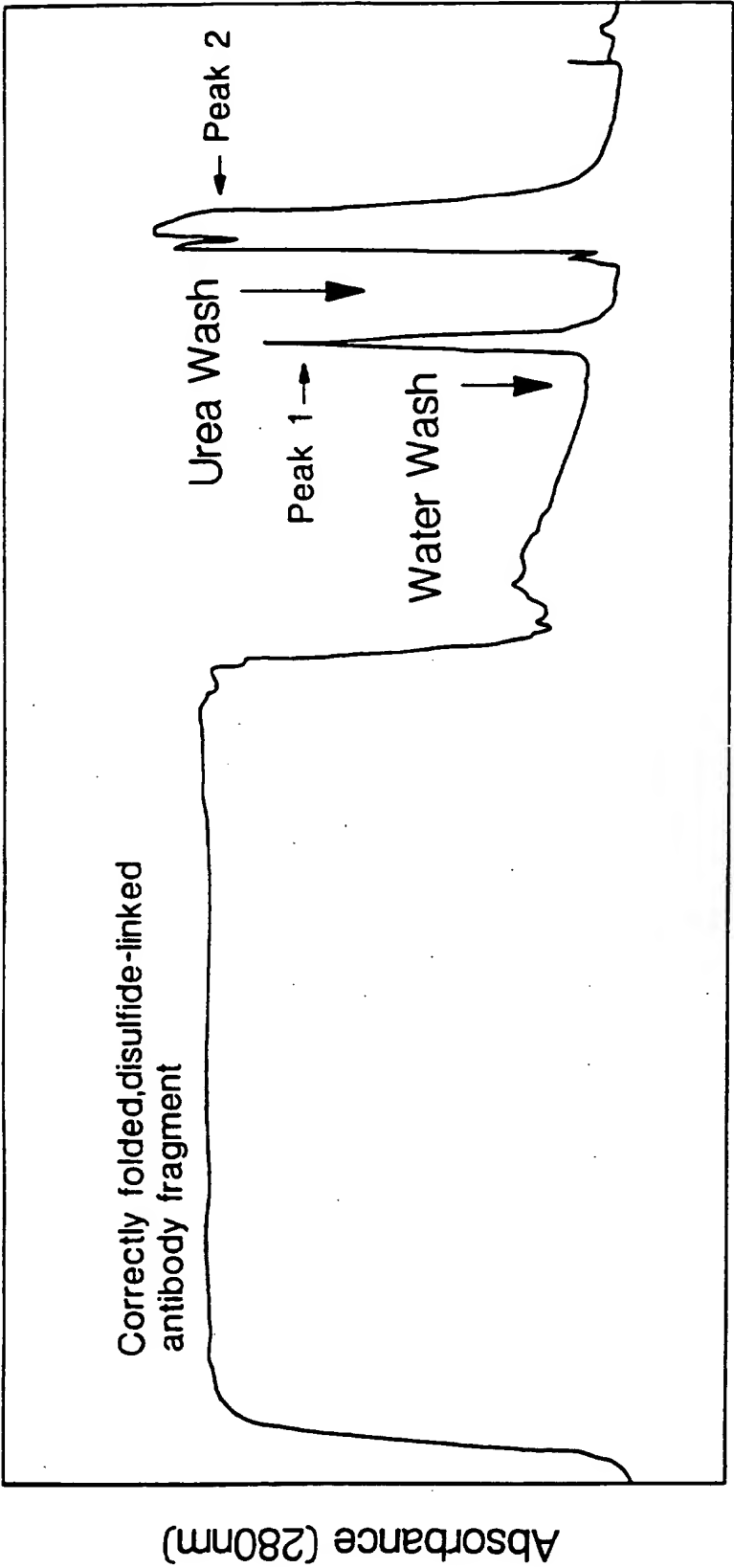
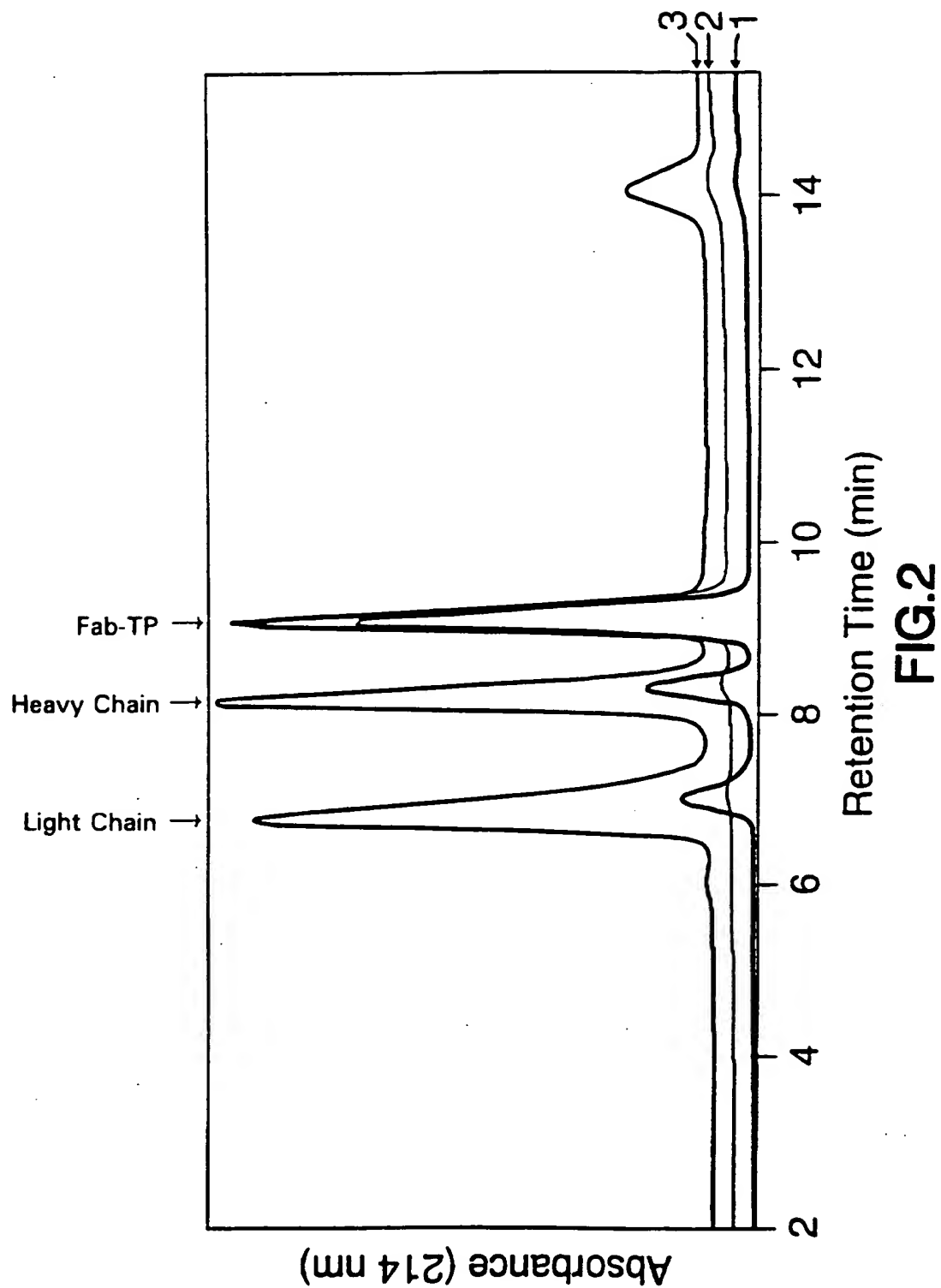
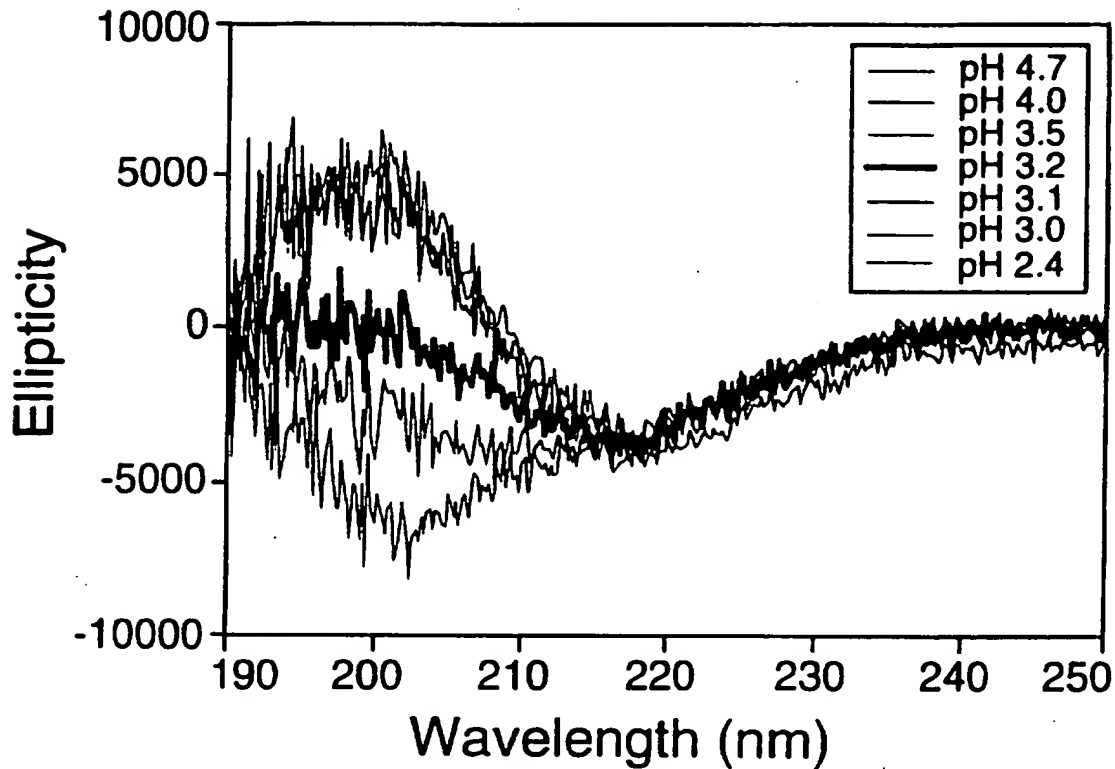
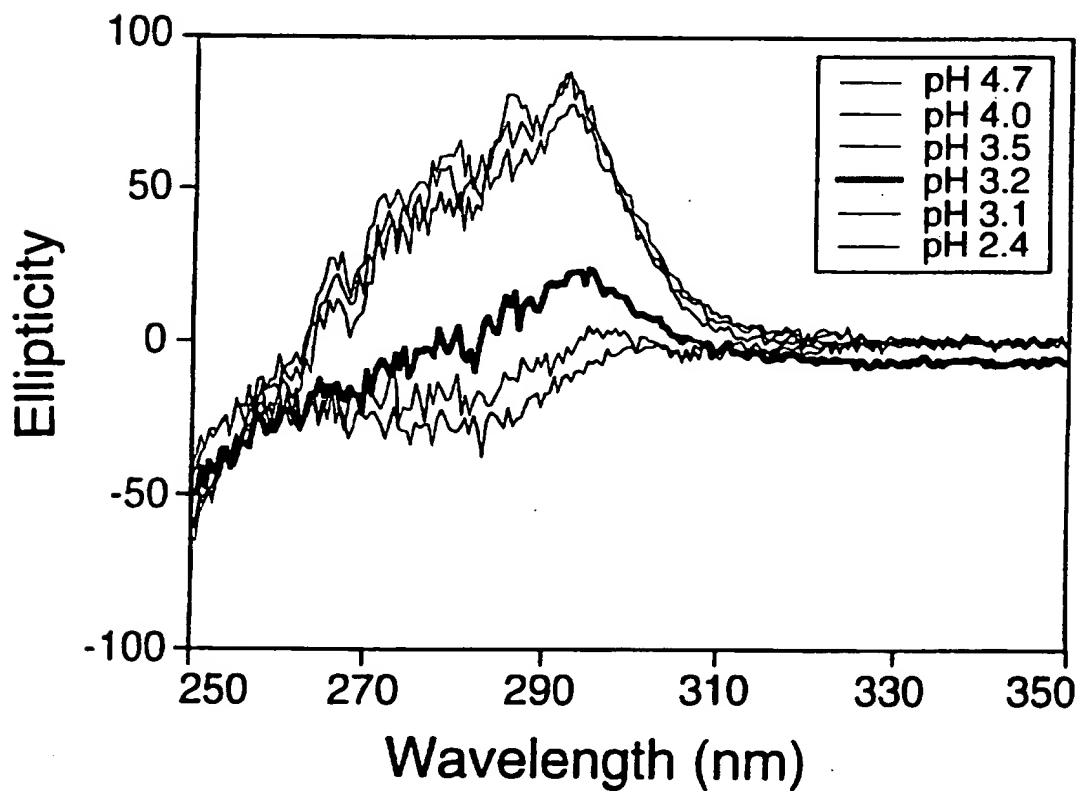


FIG.1

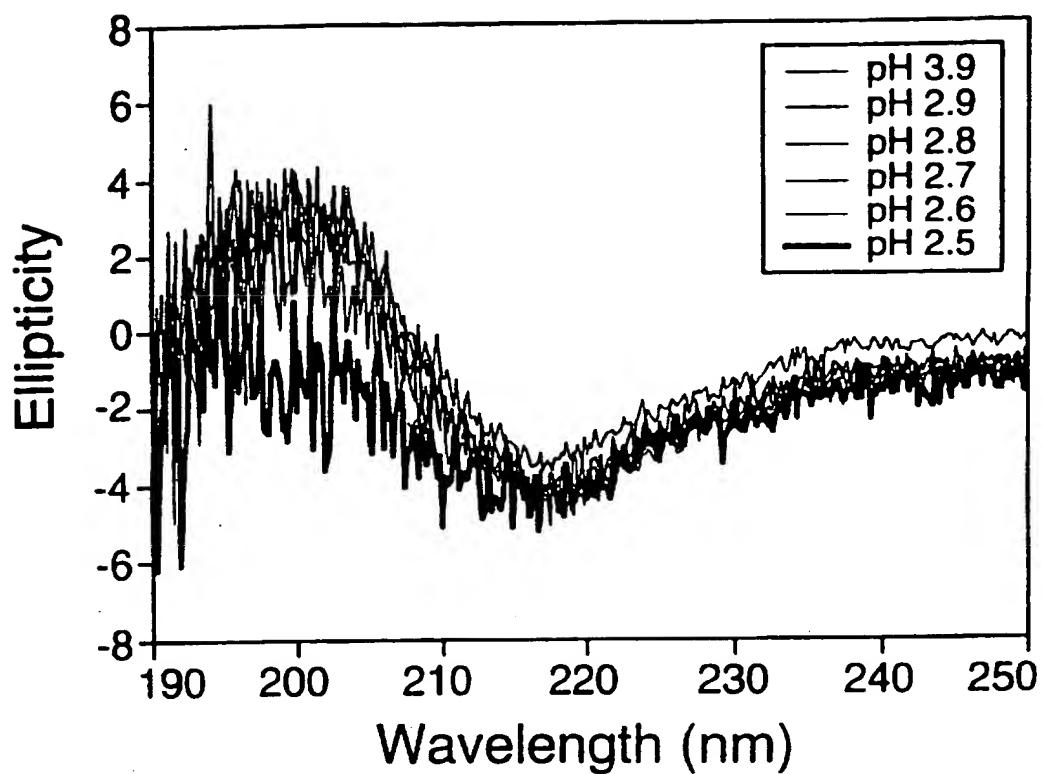
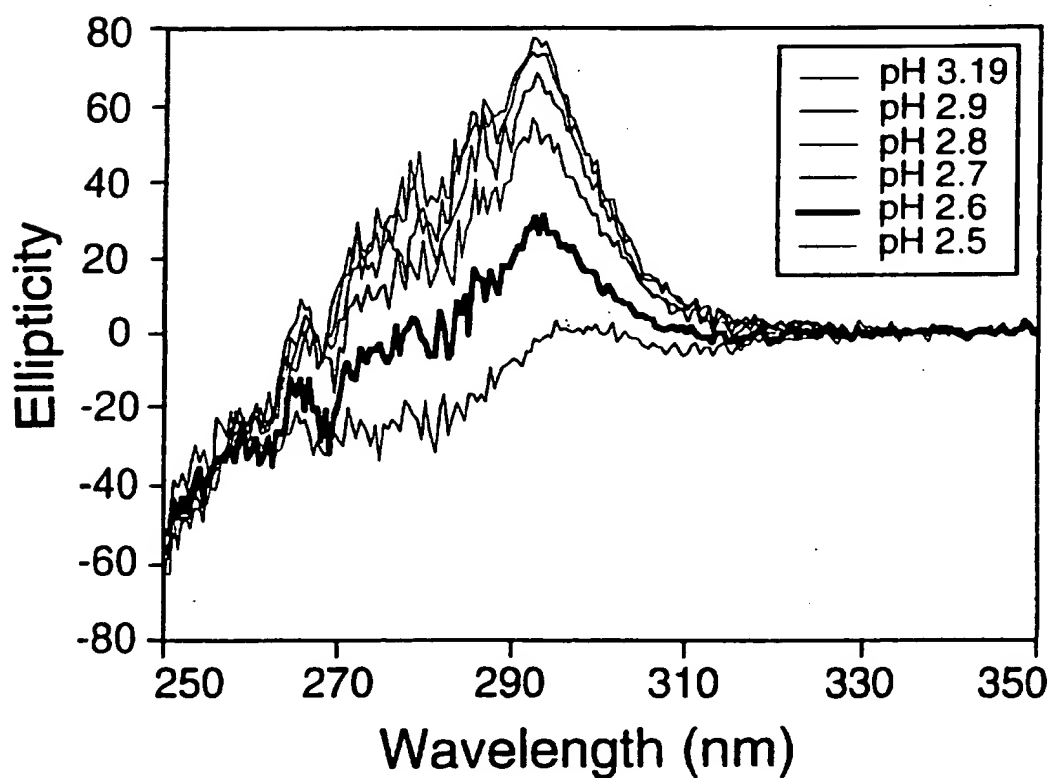
2 / 9



3 / 9

**FIG.3A****FIG.3B****SUBSTITUTE SHEET (RULE 26)**

4 / 9

**FIG.3C****FIG.3D****SUBSTITUTE SHEET (RULE 26)**

5 / 9

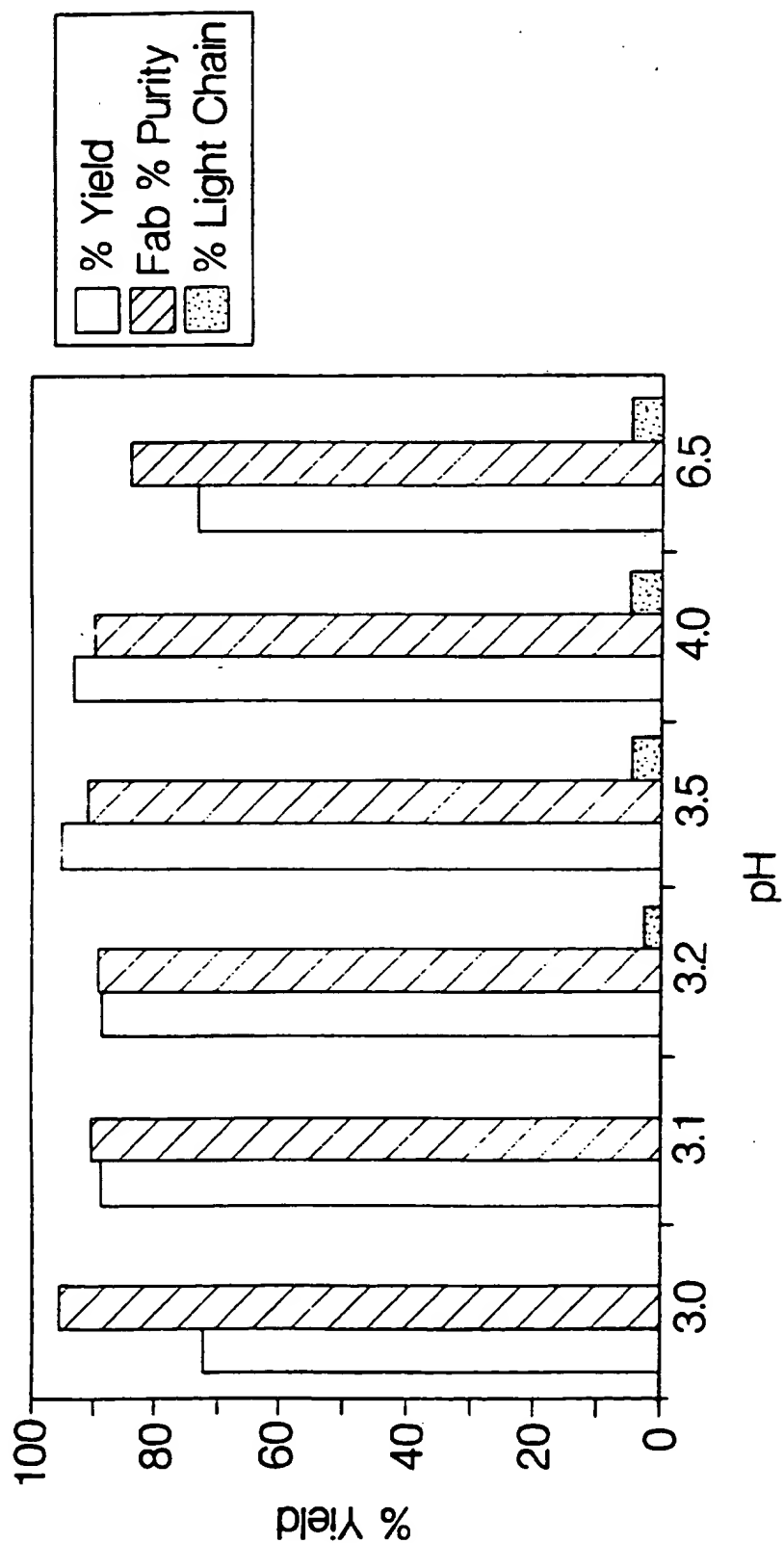


FIG.4

6 / 9

FIG.5A

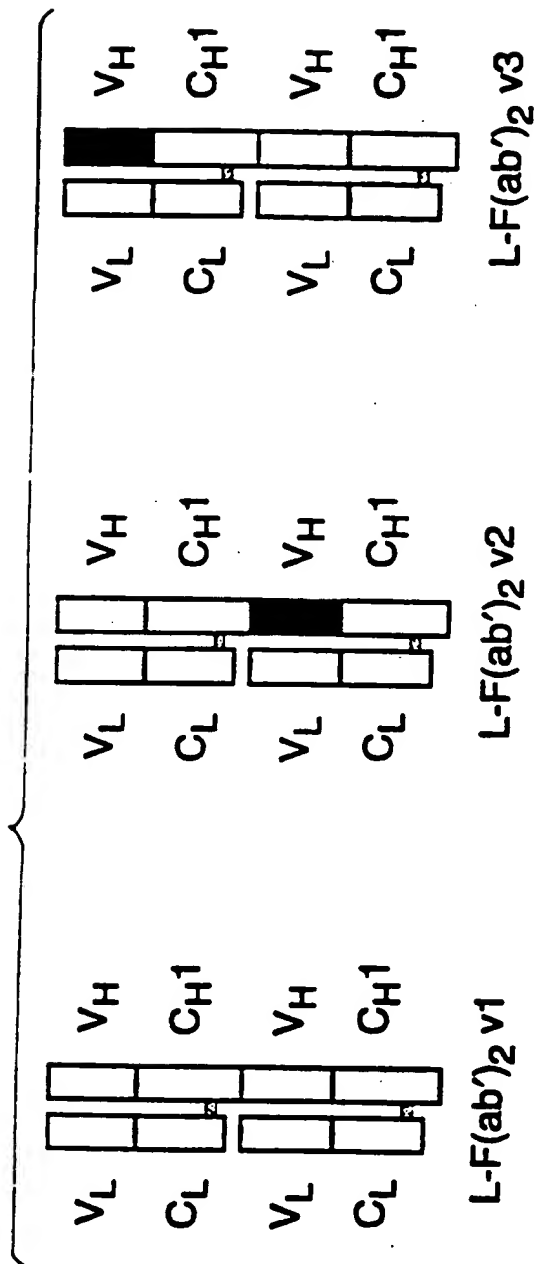
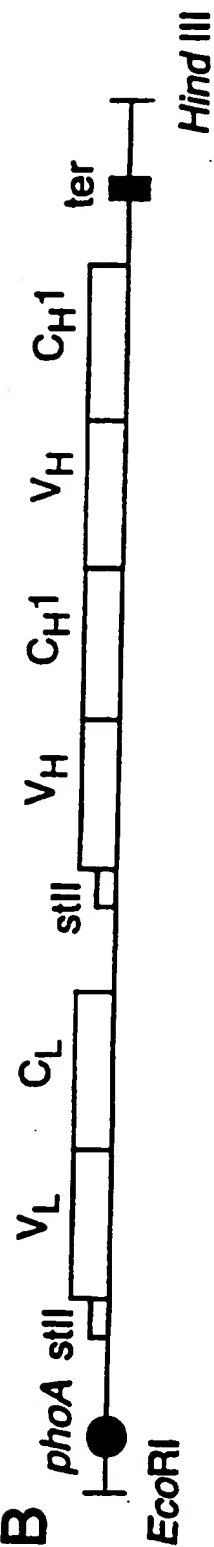


FIG.5B



7 / 9

FIG.6A

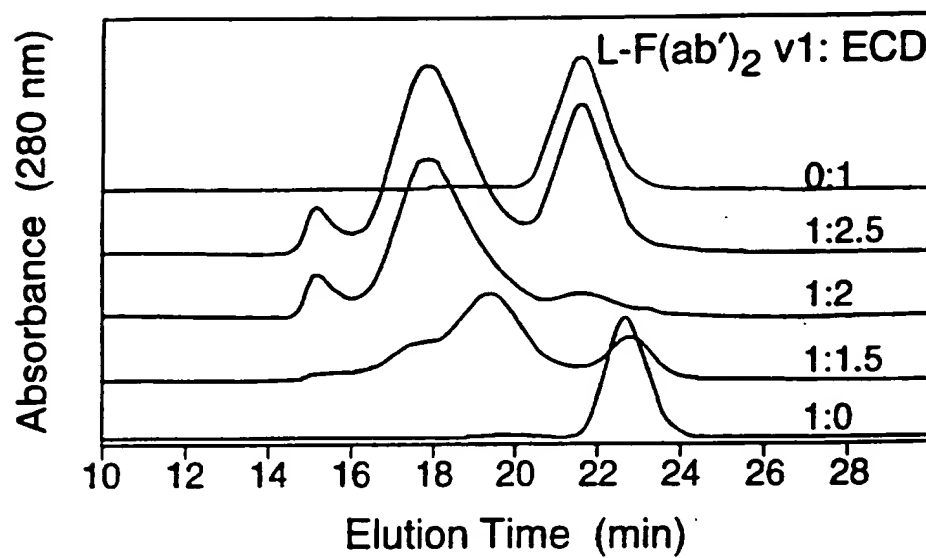


FIG.6B

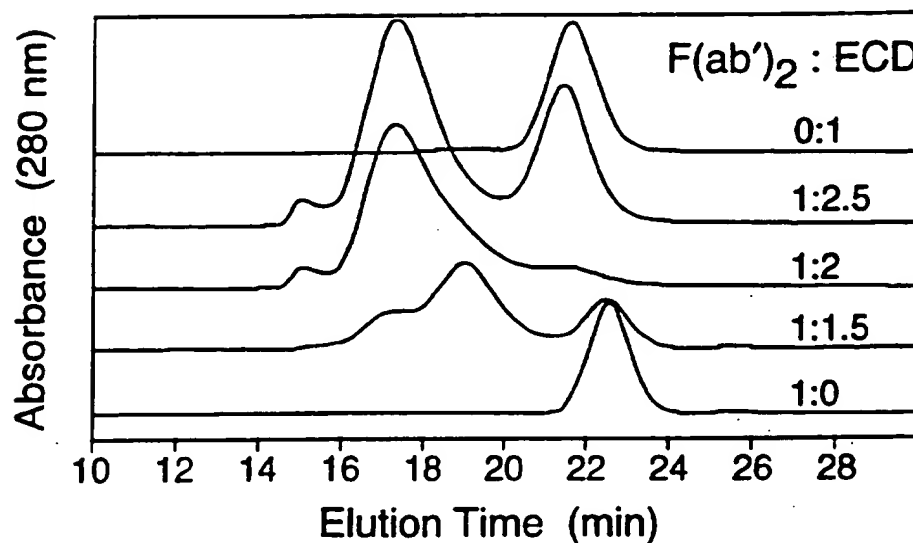
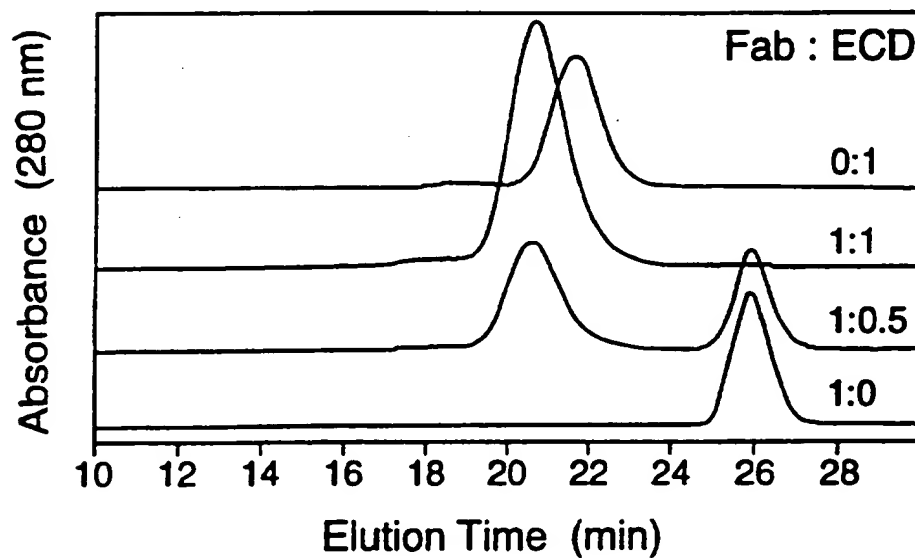


FIG.6C



8 / 9

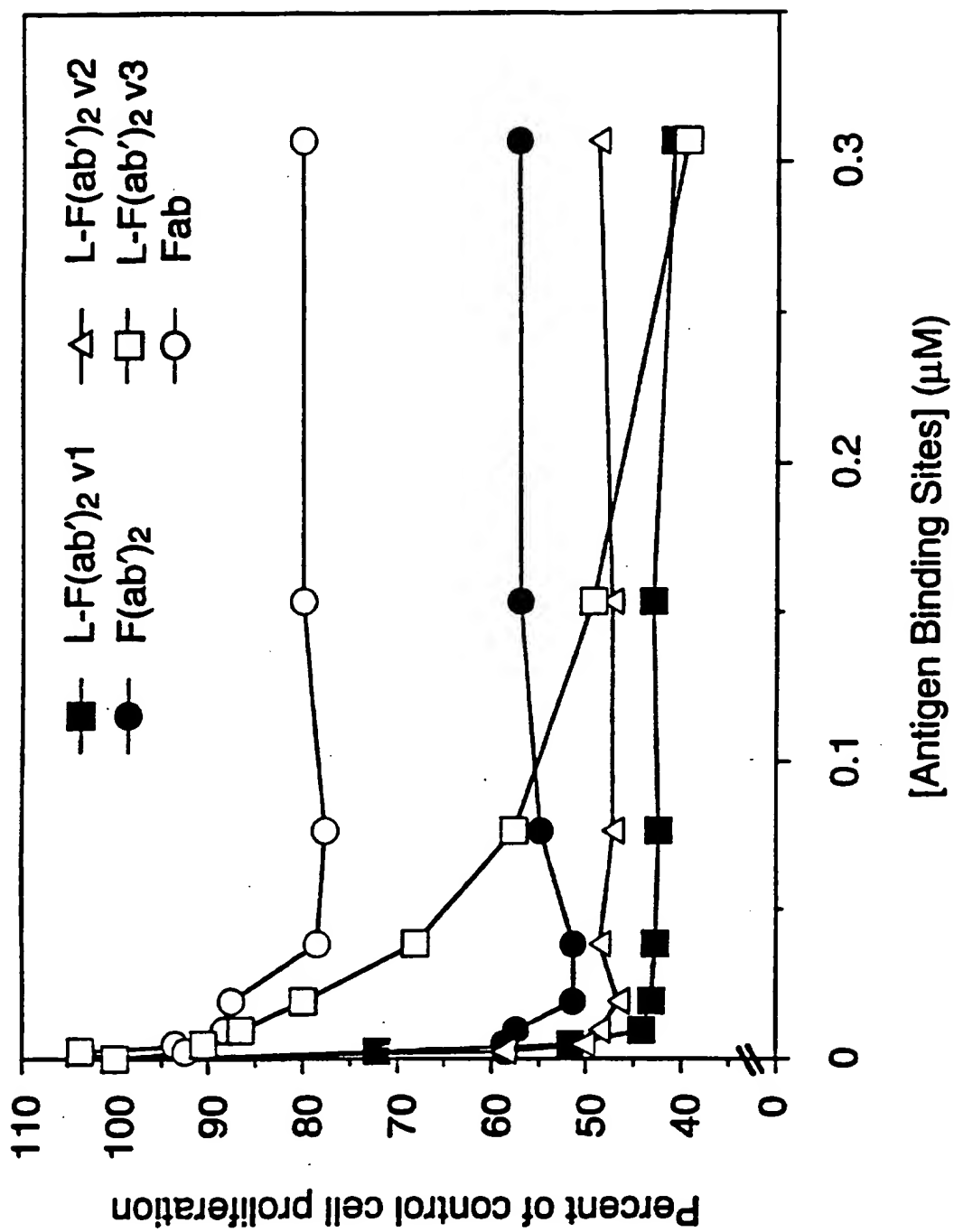


FIG.7

9 / 9

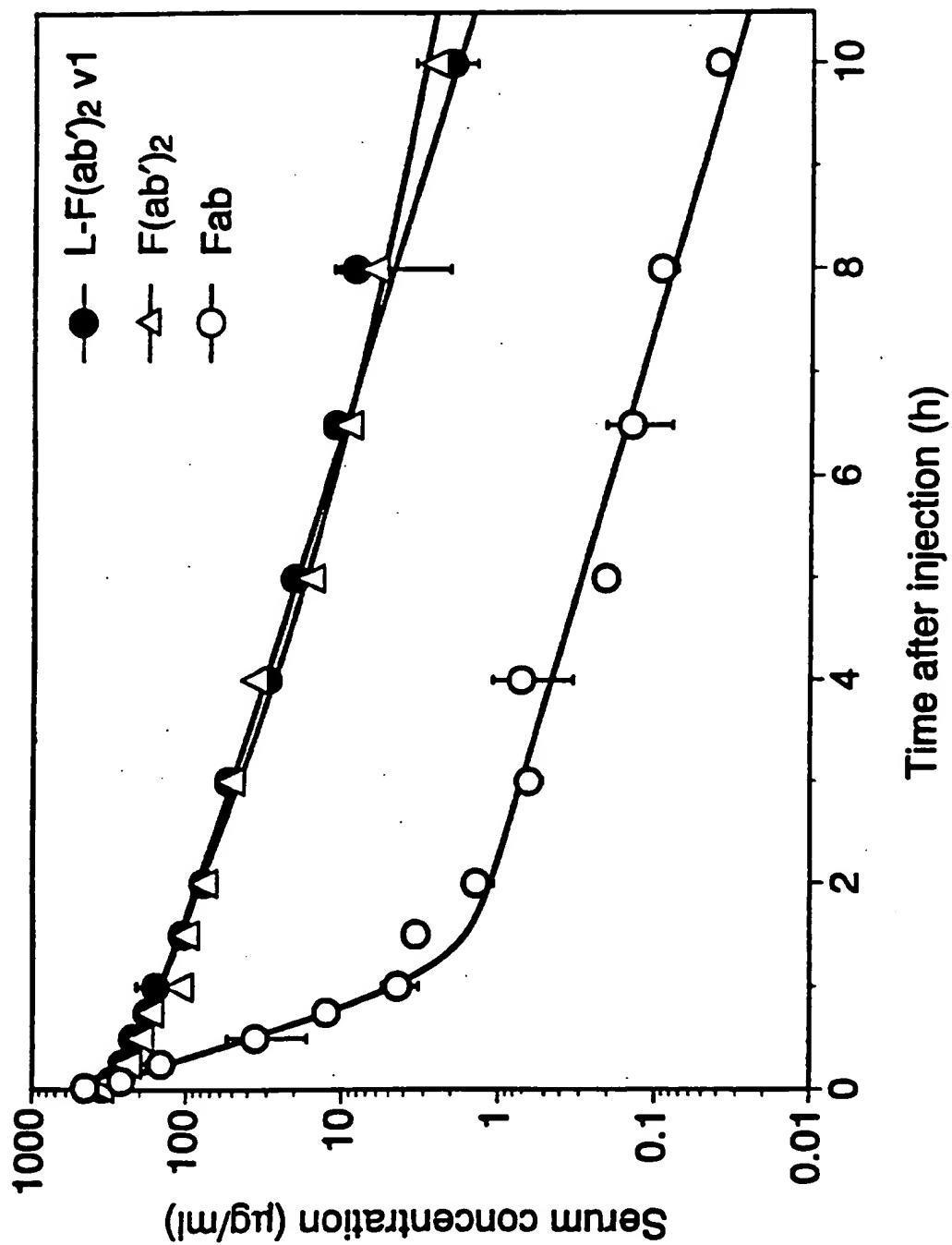


FIG.8

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/04683

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C07K1/20 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A;92 22653 (GENENTECH INC.) 23 December 1992 see examples see seq. id. nos. 22 and 24 ---	1-18
A	CANCER RESEARCH, vol. 55, no. 1, 1 January 1995, BALTIMORE, MD, USA, pages 63-70, XP002010284 M. RODRIGUES ET AL.: "Development of a humanized disulfide-stabilized anti-p185HER2 Fv-beta-lactamase fusion protein for activation of a cephalosporin doxorubicin prodrug." see material and methods --- -/--	1-18

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *&* document member of the same patent family

Date of the actual completion of the international search

6 August 1996

Date of mailing of the international search report

20.08.96

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Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 96/04683

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCONJUGATE CHEMISTRY, vol. 3, no. 2, January 1992, WASHINGTON, DC, USA, pages 126-131, XP002010285 D. NEBLOCK ET AL.: "Conjugation and evaluation of 7E3xP4B6, a chemically cross-linked bispecific F(ab') ₂ antibody which inhibits platelet aggregation and localizes tissue plasminogen activator to the platelet surface." see abstract see figure 2 see page 127, right-hand column, line 22 - line 41	1-18
A	WO,A,92 12993 (THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC.) 6 August 1992 see example 2 see claims 39,48,50,51	1-18
A	JOURNAL OF CHROMATOGRAPHY, vol. 470, no. 2, 26 May 1989, AMSTERDAM, THE NETHERLANDS, pages 385-389, XP000577281 H. ALFTHAN ET AL.: "Purification of labelled antibodies by hydrophobic interaction chromatography." see experimental	1-18
A	JOURNAL OF CELLULAR BIOCHEMISTRY, SUPPLEMENT, vol. 0, no. 17 part A, 1993, NEW YORK, NY, USA, page 50 XP000578103 D. REA ET AL.: "The rapid development of hydrophobic interaction chromatography purification of a murine monoclonal IgG2a F(ab') ₂ fragment." cited in the application see abstract X1-206	1-18
A	PROTEIN EXPRESSION AND PURIFICATION, vol. 2, no. 5-6, October 1991, SAN DIEGO, CA, USA, pages 379-384, XP000578101 G. KURZBAN ET AL.: "Purification of bovine liver rhodanese by low-pH column chromatography." see page 382, right-hand column, line 7 - page 383, left-hand column, line 2	1-18
1 6	P,A US,A,5 429 746 (SHADLE ET AL.) 4 July 1995 see the whole document	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/04683

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9222653	23-12-92	AU-B- 2250992	12-01-93
		CA-A- 2103059	15-12-92
		EP-A- 0590058	06-04-94
		JP-T- 6508267	22-09-94
		WO-A- 9404679	03-03-94

WO-A-9212993	06-08-92	US-A- 5231178	27-07-93
		AT-T- 121099	15-04-95
		AU-B- 1194792	27-08-92
		CA-A- 2098183	16-07-92
		DE-D- 69202049	18-05-95
		DE-T- 69202049	24-08-95
		EP-A- 0567554	03-11-93
		ES-T- 2071488	16-06-95
		JP-T- 6505010	09-06-94

US-A-5429746	04-07-95	AU-B- 1843395	04-09-95
		WO-A- 9522389	24-08-95
		ZA-A- 9501372	24-10-95
